Fingerprint Gas Chromatographic Analysis of Tobacco Leaf Acids

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A procedure for rapid, quantitative silylation of the hydrozyl function, developed by Makita and Wells (9, 24), has been applied to gas chromatography of C_6 $-C_{16}$ fatty acids (27), Krebs cycle acids (4), phenolic acids (1), amino acids (18, 21), bile acids (9, 13), carbohydrates (24), phenols (8, 10), and a wide range of urinary and tissue metabolites (2). Direct gas chromatography of free fatty (11) or aromatic acids (12, 26) required specially prepared columns and were limited by high boiling points of the acids. Although use of methyl esters of fatty (16) and aromatic acids (3) proved satisfactory, mixtures composed of a wide variety of fatty, polybasic and aromatic acids could not be conveniently and quantitatively esterified. Furthermore, Simmonds, Pettitt and Zlatkis were able to isolate isomerized by-products following esterification of Krebs cycle keto acids with diazomethane (20).

In this study, a procedure for gas chromatographic analysis of mixtures of aliphatic, aromatic, phenolic and certain Krebs cycle acids was developed. In particular, the method was applied to the total analysis of acids in tobacco and tobacco pyrolysates. Earlier gas chromatographic studies in this area dealt with analysis of free (7, 19, 22) and methylated acids (14, 23) from tobacco and tobacco smoke. Other studies utilized paper and column chromatography (5, 6, 15).

Since tobacco leaf composition varies as a function of curing and fermentation procedures as well as the growth history of the plant (6, 25), "fingerprint" chromatograms of the total acid fraction would be valuable for both analytical and comparative studies. Trimethylsilylation of the acid sample prior to gas chromatography proved to be most suitable for the production of such "fingerprint" chromatograms. Versatility, rapid formation and sharp, symmetrical gas chromatographic peaks are among the advantages of

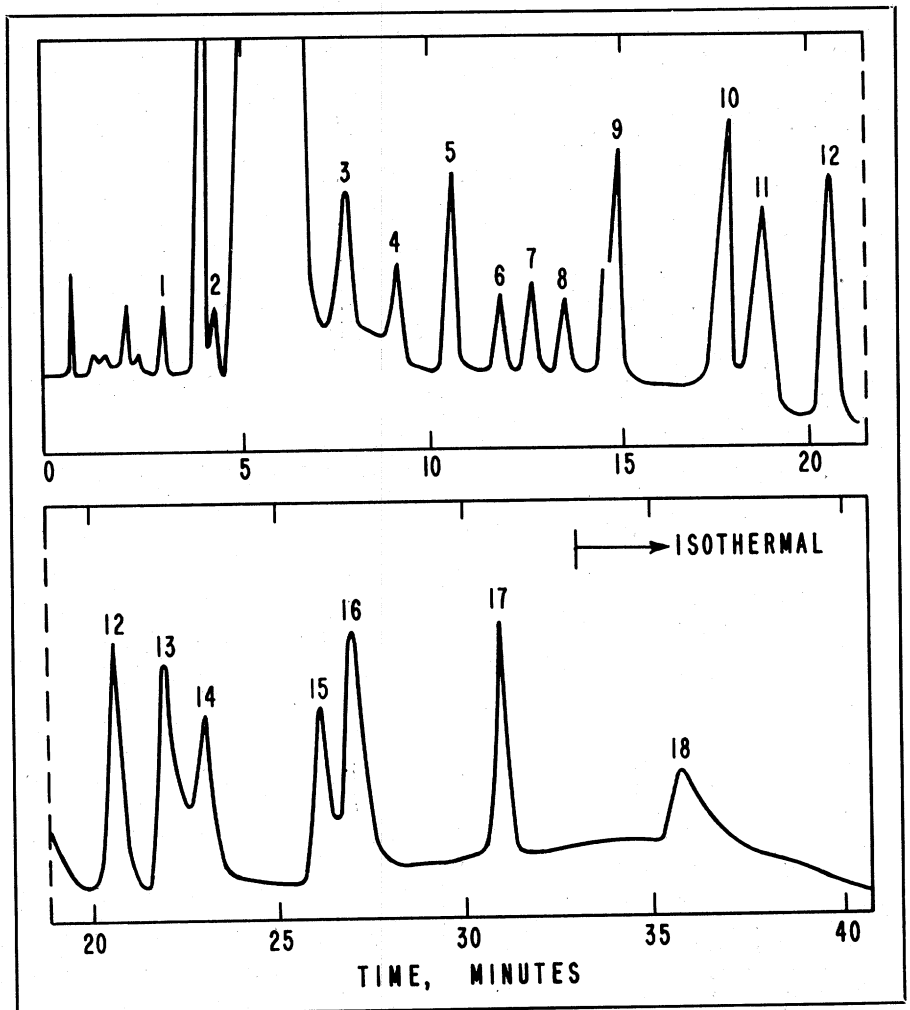

Figure 1. Typical gas chromatogram of TMS derivatives of a mixture of known acids. Peak identifications: 1 = formic, 2 = acetic, 3 = isobutyric, 4 = butyric, 5 = isovaleric, 6 = valeric, 7 = ethylbutyric, 8 = β-methylvaleric, 9 = caproic, 10 = heptanoic, 11 = malonic, 12 = octanoic, 13 = succinic, 14 = fumaric, 15 = decanoic, 16 = malic, 17 = cinnamic, 18 = citric acid. Elution temperatures are listed in Table I.

silylation. Although there are no previous reports describing the use of TMS esters of formic and acetic acids in gas chromatographic analysis, these esters form readily and produce sharp, well-resolved peaks in such analyses. Thus, in the procedure described in the present report, nearly all the significant leaf acids can be determined simultaneously with a minimum of experimental error and excellent reproducibility in elution temperatures.

Experimental

Apparatus. A Varian-Aerograph Model 200 gas chromatograph[1] equipped with a dual thermal conductivity detector and dual stainless steel columns (5-foot x 0.25-inch o.d.) was used as the basic instrument. Chromatographic separations were achieved on columns filled with 20% SE-30 on 60/80 Chromosorb W with helium as the carrier gas at a flow rate of 60 ml/min. The detector temperature was maintained at 255° C and the injector temperature at 235° C. During an analysis, column temperature was generally programmed from 70° (6° C/min) and then maintained at the upper temperature.

Silylation Procedure. Approximately 0.5 cc Tri-Sil reagent (Pierce Chemical Co.) was added to 10 mg of dry, pure acid or 30 mg of a dry mixture of 5 to 15 acids. About 30 to 50 mg of the unknown leaf acids were silylated in 0.3-0.4 cc Tri-Sil reagent. Sixty μl quantities were usually injected into the gas chromatograph. Prior to injection, the reaction mixture was vigorously agitated, and reaction was generally complete in five minutes.

Water Extraction of Tobacco Leaf Acids (15). Finely ground tobacco leaf (50 g) was extracted with 600 cc of distilled water in a Waring blender. The mixture was centrifuged, the supernatant liquid decanted and brought to pH 11 with NaOH pellets. After being washed with ether, the aqueous layer was brought to pH 2 with concd. HCL and extracted with ether. The ether layer was then dried over Na_2SO_4 and the ether removed in a rotary evaporator. The total weight of the acid residue was 1.2 g for flue-cured and 1.5 g for Turkish tobacco.

Ether Extraction of Tobacco. Finely ground Turkish tobacco leaf (100 g) was extracted 24 hours with 2 liters ether in a Soxhlet extractor. The ether solution was concentrated to 300 cc and then washed with 1% NaOH (50 cc) three times. The aqueous layer was washed three times with ether, then brought to pH 2 using concd. HCL. The acidic aqueous layer was extracted six

Table 1. Gas chromatographic elution data for trimethylsily esters of known acids[a]

Acid	Retention time, min. (isothermal at 175°C)	Elution temp., °C[b] 175°-240° at 6°/min.	Elution temp., °C[b] 70°-240° at 6°/min.
Formic[c]			88°
Acetic[c]			96°
Isobutyric			112°
Isovaleric[d] and *trans*-crotonic			128°
Butyric			119°
Valeric			134°
Ethylbutyric			137°
β-Methylvaleric			142°
Caproic[d]			
lactic (peak 1)[e]	2.1		149°
Oxalic	2.5		160°
Furoic	2.7		
Heptanoic			165°
Malonic	3.6		172°
Octanoic			180°
Benzoic	4.5		
Succinic[d,f]			
Phenylacetic			
Nicotinic	5.5	205°	187°
Isonicotinic			
Maleic			
Fumaric[d,f]			
Citraconic	6.4		194°
Nonanoic			
Hydrocinnamic		216°	
Lactic, (peak 2)[e]	7.8		200°
Decanoic			207°
Adipic		222°	
Salicylic		227°	
Malic	12.2		
Tartaric			222°
p-Hydroxybenzoic		239°	
Lauric,[d,f] cinnamic[d,f]		230°	235°

	Retention time at upper temp. limit (240°C)[g]	
1 2-Benzene dicarboxylic	0.7 min.	
1,3-Benzene dicarboxylic	1.8 min.	0.5 min.
1 4-Benzene dicarboxylic,[d,f] azelaic	2.6 min.	1.4 min.
Diphenyl acetic	2.8 min.	
Myristic	3.2 min.	2.4 min.
Citric		2.7 min.
Benzilic	4.2	
Palmitic	7.8	14.4

[a] *Experimental Section lists the chromatography conditions.*

[b] *Linear temperature programming at 6°/min. Elution temperature is reproducible to ±2° for different sets of runs.*

[c] *Acid must be dry before silylation.*

[d] *Same retention time.*

[e] *Lactic acid (TMS) derivative has two peaks.*

[f] *When several acid (TMS) esters had the same retention time, the peaks were collected for infrared spectral and thin-layer chromatographic analysis for proper identification.*

[g] *After temperature was programmed to 240°, it was then held at 240°. Retention time was computed by setting the time at the beginning of the isothermal (240°C) portion of the chromatogram to 0.0 min.*

[1] *Mention of trade or company names is for identification only and does not imply endorsement by the department.*

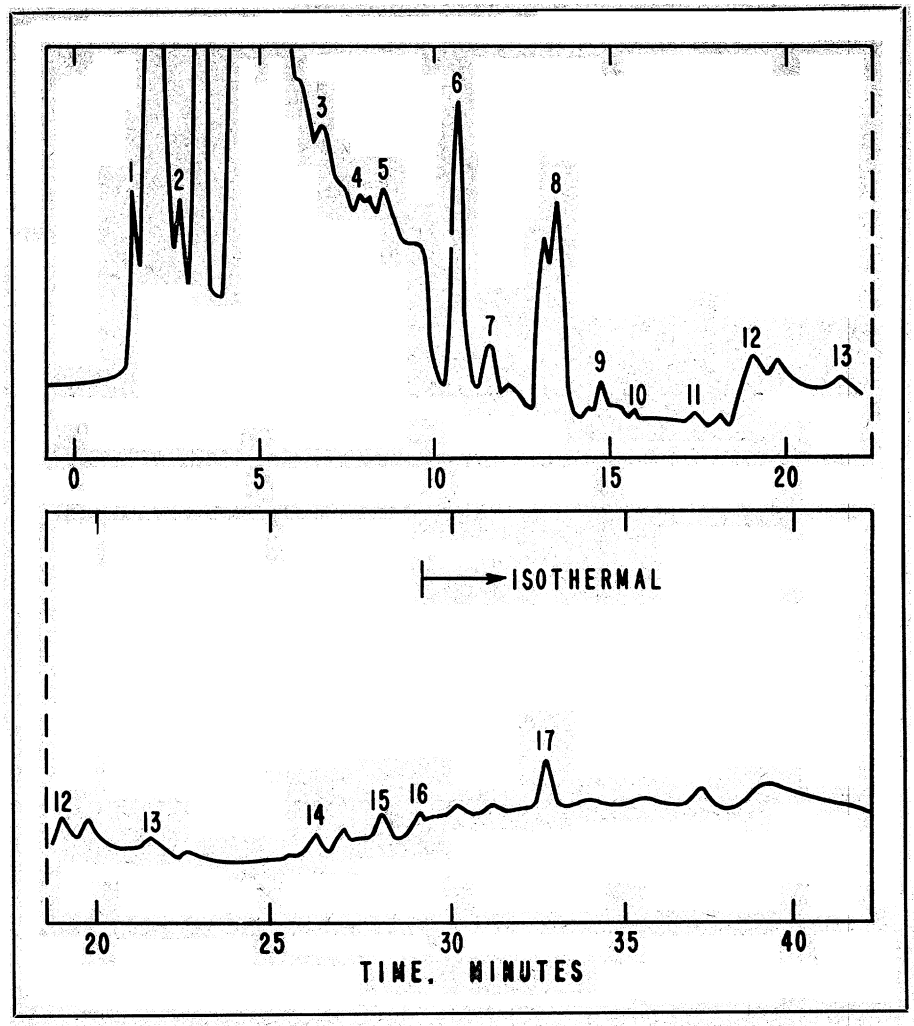

Figure 2. Typical gas chromatogram at TMS derivative of water extracted acids from flue-cured tobacco. Peak identifications: 1 = formic 8X, 2 = acetic 8X, 3 = isobutyric 2X, 4 = butyric 2X, 5 = isovaleric 2X, 6 = valeric 4X, 7 = ethylbutyric 2X, 8 = caproic 4X, 9 = oxalic 1X, 10 = heptanoic 1X, 11 = malonic 1X, 12 = octanoic 1X, 13 = fumaric 1X, 14 = tartaric 1X, 15 = cinnamic 1X, 16 = linoleic 1X, 17 = citric 1X. Elution temperatures listed in Table 1.

times with ether and the combined ether layers were dried. The ether was removed. Weight of the "acid residue," which contained large quantities of ethanol, was 26 g. Formic acid was the predominant acid. To facilitate analysis of the nonvolatile acids present, 3 cc of the residue was evaporated to 0.10 cc before silylation.

Analysis of Unknown Acid Mixtures. In all analyses, gas co-chromatography with mixtures of known acids, as well as peak collection and subsequent infrared spectral studies, was used to confirm identifications.

Results and Discussion

General Procedure. Trimethylsilyl (TMS) esters of mixtures of aliphatic, Krebs cycle, aromatic and phenolic acids gave chromatograms with sharp, symmetrical peaks and excellent resolution (**Figure 1**). Infrared spectra were taken immediately after collection of the individual peaks and again after 24 hours. The esters were observed to hydrolyze upon standing; spectra taken 24 hours after collection were identical with those of the free acids. No absorption bands corresponding to those reported for silyl esters or ethers (10, 17) were seen in the spectra. Facile hydrolysis of the trimethylsilyl esters makes possible unambiguous infrared spectra as well as recovery of the parent acid (17).

Table 1 summarizes elution data obtained from mixtures of TMS derivatives of known acids. Lactic acid, interestingly enough, gives a characteristic doublet of peaks as a result of mono- and disilylation (carboxyl and hydroxyl groups). This property has proven useful for rapid differentiation of lactic acid from caproic acid whose TMS derivatives elutes at the same time.

In general, the unsaturated analog of an acid appeared to have a longer retention time than the saturated acid. Branched chain isomers of the C_1–C_6 fatty acids had shorter retention times than the straight chain acids. Various isomers of the C_1–C_7 fatty acid TMS derivatives showed distinct, well-resolved peaks. The TMS ester of propionic acid eluted with pyridine, the silylation solvent, and its presence in acid mixtures was thus masked. The free acid could be detected, however, by gas chromatography of unesterified samples of the acid mixture. Alternately, another solvent could be used in the silylation reaction. The 1,2-, 1,3-, and 1,4-benzene dicarboxylic acid TMS derivatives were resolved into three distinct peaks on the column used. E. R. Blakely (1) has also discussed the value of trimethylsilyl esters for the separation and identification of many closely related phenolic acid isomers.

When several TMS esters had identical retention times, thin layer chromatography and infrared spectra of the collected peaks were utilized for positive identification. If infrared data were insufficient for characterization, the parent acid[2] could be analyzed further by other techniques.

Despite the vigorous conditions reported for silylation of phenol, *o*- and *p*-cresols (10), they appeared to react rapidly with the Tri-Sil reagent at room temperature upon agitation. The order of the retention times agreed with those reported by Nelson and Smith (10).

By programming the temperature of the columns from 70° to 240° C at 6°/min and then maintaining it at 240° C, one can analyze virtually the complete range of C_1–C_{16} acids (as their TMS esters) in mixtures of acids. If only nonvolatile acids are present in the mixture the samples can be run from 175° to 240° C at 6°/min and then maintained at 240° C or isothermally at 175° C as shown in **Table 1.** Although retention times varied during linear temperature programming (70° to 240° at 6°/min) as evidenced in **Figure 1, 2, 3 and 4,** elution temperatures were reproducible to ±2° C in all cases. Thus, "fingerprint chromatograms" of high reproducibility could be obtained.

No intensive quantitative studies of mixtures of the trimethylsilyl derivatives of the acids have been undertaken at present. However, under isothermal conditions a distinct linear relationship between peak area and quantity injected appeared to hold true. Earlier quantitative studies of trimethylsilyl derivatives of amino acids had indicated excellent internal consistency

[2] *Hydrolysis of the trimethylsilyl ester to the parent acid occurs at room temperature after 24 hours.*

(21) as well as good quantitative correlations (21)

Application to Tobacco Acids. Acids from water extraction of flue-cured and Turkish tobacco leaf were silylated and analyzed by gas chromatography. **Figures 2 and 3** illustrate the type of "fingerprint" chromatograms obtained. No quantitative correlations were made between the different acids in as much as the composition of the extracted acids appeared to be dependent on the volumes of the aqueous layers present in different stages of the extraction procedure. Variations in solubility and accessibility of the different leaf acids to water during the initial extraction, as well as the partition coefficients between water and ether of such acids and their sodium salts were factors likely to influence final composition.

Turkish tobacco acids were alternately obtained by continuous ether extraction of the leaf for 24 hours. Care was taken to keep volumes of the aqueous layer to a minimum during product isolation. To facilitate analysis of the acid residue (13 g/50 g tobacco), ethanol and formic acid which were present in large amounts, were removed before silylation. **Figure 4** represents a "fingerprint gas chromatogram" of the ether extracted acids after 30-fold concentration of the sample.

Table 2 summarizes the qualitative results from gas chromatographic analysis of leaf acids. In all cases, gas co-chromatography with mixtures of knowns and infrared spectra of the collected peaks were utilized to confirm identifications. The tobacco leaf acids found were in general agreement with earlier reports. However, previously unreported cinnamic acid was identified in both flue-cured and Turkish tobaccos.

Figures 2, 3 and 4 represent the general appearance of the resulting gas chromatograms, and illustrate in some measure the versatility and completeness of this procedure for comprehensive analysis of mixtures of naturally-occuring acids. Also, the procedure is valuable whenever accurate comparisons between systems of acid mixtures are required. In particular, we can now simultaneously analyze formic, acetic and volatile C_4–C_6 acids along with a wide range of C_6–C_{22} aliphatic and aromatic acids. Acetic, formic, and volatile C_4–C_6 acids have previously required more laborious and time consuming methods of analysis. Moreover, errors that would be introduced in the separation of lower fatty acids, Krebs cycle acids, and

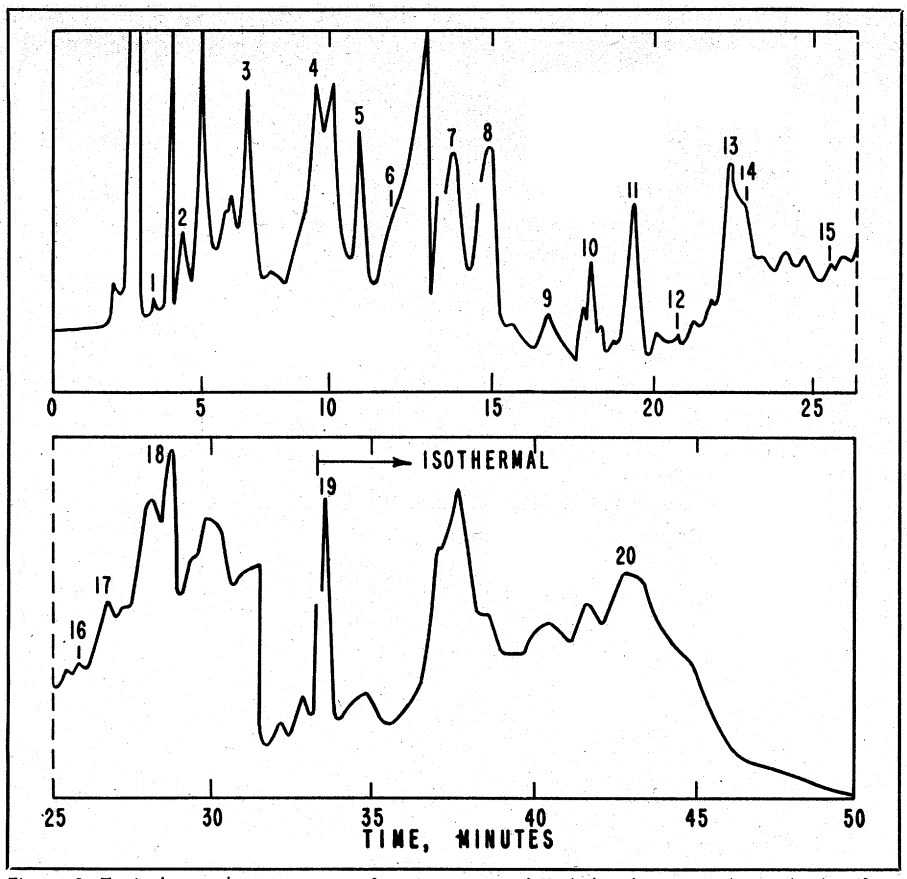

Figure 3. Typical gas chromatogram of water extracted Turkish tobacco acids. Peak identifications: 1 = formic 8X, 2 = acetic 8X, 3 = isobutyric 8X, 4 = butyric 8X, 5 = isovaleric 8X, 6 = valeric 8X, 7 = 3-methylvaleric 32X, 8 = caproic 2X, 9 = oxalic 1X, 10 = heptanoic 1X, 11 = malonic 1X, 12 = octanoic 1X, 13 = succinic 1X, 14 = fumaric 1X, 15 = decanoic 1X, 16 = malic 1X, 17 = tartaric 1X, 18 = cinnamic 1X, 19 = citric 2X, 20 = palmitic 1X,. Elution temperatures listed in Table I.

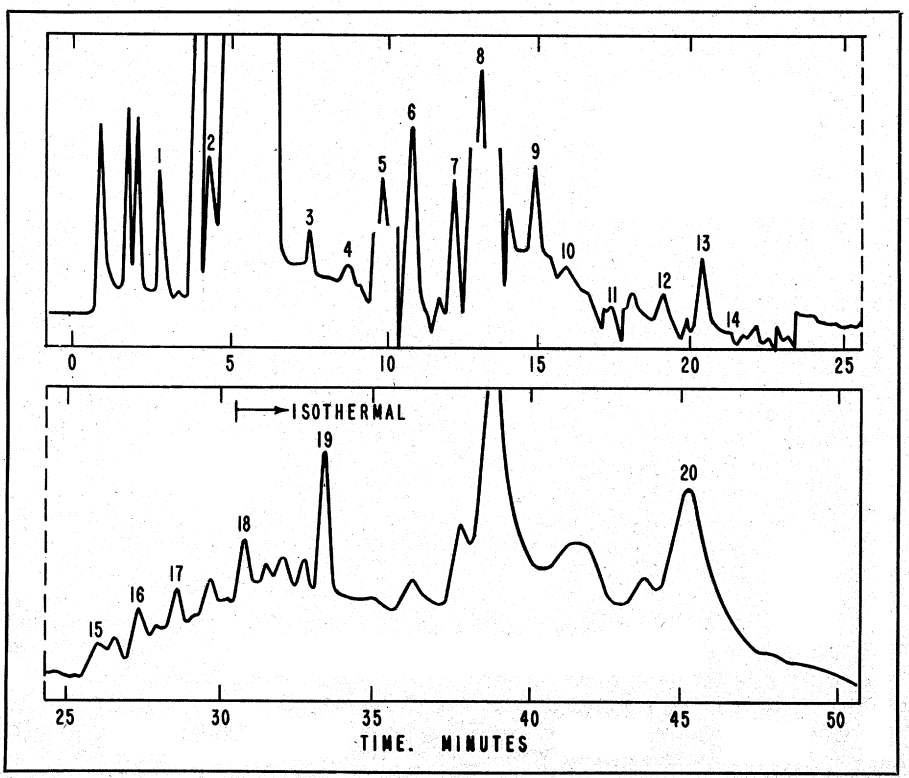

Figure 4. Typical gas chromatogram of ether extracted Turkish tobacco acids. Peak identifications: 1 = formic 32X, 2 = acetic 16X, 3 = isobutyric 4X, 4 = butyric 4X, 5 = isovaleric 16X, 6 = valeric 4X, 7 = ethylbutyric 1X, 8 = β-methylvaleric 32X, 9 = caproic 1X, 10 = heptanoic 1X, 11 = malonic 1X, 12 = octanoic 1X, 13 = succinic 1X, 14 = fumaric 1X, 15 = malic 1X, 16 = α tartaric 1X, 17 = cinnamic 1X, 18 = linoleic 1X, 19 = citric 1X,, 20 = palmitic 1X. Elution temperatures listed in Table I.

Table 2. Tobacco leaf acids identified from chromatograms of their trimethylsilyl esters[a,b]

Acids	Water extracted acids from flue-cured tobacco leaf[c]	Water extracted acids from Turkish tobacco leaf	Ether extracted acids from Turkish tobacco leaf
Formic	+	+	+
Acetic	+	+	+
Isobutyric	+	+	+
Butyric	+	+	+
Isovaleric	+	+	+
Valeric	+	+	+
β-Methylvaleric	+	+	+[d]
Ethylbutyric	+	—	+
Caproic	+	+	+
Oxalic	+	+	+
Heptanoic	+	+	+
Malonic	+	+	+
Octanoic	+	+	+
Succinic	+	+	+
Fumaric	+	+	+
Malic	—	+	+
Tartaric	+	+	+
Cinnamic[e]	+	+	+
Citric	+	+	+
Palmitic	—	+	+
Linoleic[f]	?	?	?

[a] *About 50 mg of the acid mixture was silylated with 0.4 cc. Tri-Sil Reagent for each chromatographic run.*

[b] *Data were taken from sets of chromatograms of the trimethylsilyl derivatives of the acids; identifications were confirmed by co-chromatography with known acids, and infrared spectral analysis of the collected peaks.*

[c] *C1-C6 acids were present in 4-8 times the quantity of the other acids.*

[d] *Turkish tobacco acids have markedly prominent quantities of β-methylvaleric acid; see reference 22.*

[e] *Previously unreported.*

[f] *Not positively identified.*

aromatic acids can be minimized by the simultaneous analysis of all the acids.

At present, we are applying this procedure to the analysis of acid fractions from the pyrolysis of tobacco leaf acids at different temperatures as well as to analysis of pyrolysis products of various acid mixtures.

Summary

Acids, extracted from tobacco leaf by conventional means, were converted to their trimethylsilyl esters prior to analysis by gas chromatography. Volatile acids, including formic and acetic, certain Krebs cycle acids, aromatic and fatty acids were all amenable to detection in a single chromatographic analysis. The method, essentially qualitative, can be used to rapidly screen mixtures of tobacco leaf acids for purposes of comparison. Use of the method resulted in the identification of cinnamic acid in tobacco leaf for the first time.

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Literature Cited

1. Blakley, E. R. Gas chromatography of phenolic acids. *Anal. Biochem.* 15: 350-354. 1966.
2. Dalgliesh, C. E., E. C. Horning, M. G. Horning, K. L. Knox and K. Yarger. A gas-liquid-chromatographic procedure for separating a wide range of metabolites occurring in urine or tissue extracts. *Biochem. J.* 101: 792-810. 1966.
3. Funasaka, W., T. Kojima and H. Hirono. Gas chromatographic analysis of mixtures of benzene polycarboxylic acids. *Bunseki Kagaku* 13: 1116-1120. 1964.
4. Horii, Z., M. Makita and Y. Tamura. Gas-liquid chromatographic separation of acids of Krebs cycle as trimethylsilyl derivatives. *Chem. Ind.* 1494. 1965.
5. Jarboe, C. H., and A. D. Quinn. Low temperature column chromatography of tobacco acids. *Tob. Sci.* 4: 168-171. 1960.
6. Johnstone, R. A. W. and J. R. Plimmer. The chemical constituents of tobacco and tobacco smoke. *Chem. Rev.* 59: 885-936. 1959.
7. Kaburaki, Y. and Y. Sato. Gas chromatographic analysis of volatile organic acids in tobacco leaves. *J. Agr. Chem. Soc. Japan* 36: 865-869. 1962.
8. Langer, S. H., P. Pantages and I. Wender. Gas-liquid chromatographic separation of phenols as trimethylsilyl ethers. *Chem. Ind.* 1664-1665. 1958.
9. Makita, M. and W. W. Wells. Quantitative analysis of fecal bile acidas by gas-liquid chromatography. *Anal. Biochem.* 5: 523-530. 1963.
10. Nelson, P. F. and J. G. Smith. The gas chromatography and infrared spectroscopy of trimethylsilyl ethers of some phenols related to lignin and ellagitannins. *Tappi.* 49: 215-218. 1966.
11. Nikelly, J. G. Gas chromatography of free fatty acids. *Anal. Chem.* 36: 2244-2248. 1964.
12. Niedermayer, A. O. Determination of phenylacetic acid in penicillin fermentation media by means of gas chromatography. *Anal. Chem.* 36: 938-939. 1964.
13. Okishio, T., P. P. Nair and M. Gordon. Studies on bile acids. The microquantitative separatio of cellular bile acids by gas-liquid chromatography. *Biochem. J.* 102: 654-659. 1967.
14. Quin, L. D. and M. E. Hobbs. Analysis of the nonvolatile acids in cigarette smoke by gas chromatography of their methyl esters. *Anal. Chem.* 30: 1400-1405. 1958.
15. Resnick, F. E., L. A. Lee and W. A. Powell. Chromatography of organic acids in cured tobacco. *Anal. Chem.* 27: 928-931. 1955
16. Robb, E. W. and J. J. Westbrook III. Preparation of methyl esters for gas liquid chromatography of acids by pyrolysis of tetramethylammonium salts. *Anal Chem.* 35: 1644-1647. 1963.
17. Robiette, A. G. and J. C. Thompson. The infrared spectra of some silyl esters. *Spectrochim Acta.* 21: 2023-2030. 1965.
18. Ruehlmann, K. and G. Michael Analysis by chromatography in the gaseous phase of silylated derivatives of amino acids. *Bull*

Soc. Chim. Biol. 47: 1467-1475. 1965.
19. Schmeltz, I., R. L. Miller and R. L. Stedman. Gas chromatographic study of the steam-volatile fatty acids of various tobaccos. *J. Gas Chromatog.* 1 (8): 27-28. 1963.
20. Simmonds, P. G., B. C. Pettitt and A. Zlatkis. Esterification, identification, and gas chromatographic analysis of Krebs cycle keto acids. *Anal. Chem.* 39: 163-167. 1967.
21. Smith, E. D. and H. Sheppard, Jr. Quantitative gas chromatography of amino-acids as trimethylsilyl derivatives. *Nature* 208: 878-880. 1965.
22. Stedman, R. L., D. Burdick and I. Schmeltz. Composition studies on tobacco. XVII. Steam-volatile acidic fraction of cigarette smoke. *Tob. Sci.* 7: 166-169. 1963.
23. Swain, A. P. and R. L. Stedman. Analytical studies on the higher fatty acids of tobacco. *J. Assoc. Agr. Chemists.* 45: 536-540. 1962.
24. Sweeley, C. C., R. Bentley, M. Makita and W. W. Wells. Gas-liquid chromatography of trimethylsilyl derivatives of sugars and related substances. *J. Am. Chem. Soc.* 85: 2497-2507. 1963.
25. Wynder, E. L. and D. Hoffmann. Advances in Cancer Research, Vol. 8, A. Haddow and S. Weinhouse, ed. Academic Press, New York, 1964, pp 341-345.
26. Vandenheuvel, W. J. A. The gas-liquid chromatographic behavior of some closely related disubstituted phenylacetic acids. *J. Chromatog.* 25: 29-33. 1966.
27. Wood, R. D., P. K. Raju and R. Reiser. Gas-liquid chromagraphic analysis of monoglycerides as their trimethylsilyl ether derivatives. *J. Am. Oil Chem. Soc.* 42: 161-165. 1965.